IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventor:		Tariq M. RANA
Application Number	:	10/722,176
Filing Date	:	November 24, 2003
Title	:	DELIVERY OF siRNAs
Attorney Docket No.	:	20336-00016 (previously UMY-059)
Examiner	:	Kimberly Chong
Group Art Unit	:	1635

Commissioner for Patents
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Sir:

DECLARATION OF TARIQ M. RANA UNDER 37 C.F.R. §1.132

I, Tariq M. Rana, hereby make the following declaration:

1. I am the inventor of the invention described in U.S. patent application 10/722,176 filed November 24, 2003 ("the '176 application"), and U.S. provisional patent application 60/430,520, filed on November 26, 2002 ("the '520 application").

2. The '176 application as filed (and published as US 2004/0204377 A1 on October 12, 2004) describes the use of a delivery mixture comprising a dendrimer and a nucleic acid capable of mediating RNA interference in Example 1 (paragraph [0102] of the published application and FIGS. 1A and 1B), Example 2 (paragraph [0103] of the published application and FIG. 2), and Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I).

3. The corresponding description of the use of a delivery mixture comprising a dendrimer and a nucleic acid capable of mediating RNA interference can be found in U.S. provisional patent application no. 60/430,520 in Example 2 (page 19 line 23 to page 20, line

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31 and FIGS. 1A and 1B), Example 3 (page 21, lines 1-21 and FIG. 2), and Example 8 (page 24, line 29 to page 25, line 14 and FIGS. 9A –9I).

4. Transfection using the delivery medium comprising a dendrimer and a nucleic acid capable of mediating RNA interference was performed in a standard volume of 1 ml in 60 mm plates (e.g., Example 7) and Lipofectamine™ (page 4 of manufacturer's instructions for use, copy submitted with accompanying Supplementary Information Disclosure Statement).

5. Microscopic examination of transfected cells showed distribution of labeled siRNA in both cytoplasm and the nuclear region for both Lipofectamine™ and PAMAM dendrimer, although there was an indication of a difference in routings ('176 application, paragraphs 110 and 111).

6. The substance of the '176 application related to a delivery medium comprising a dendrimer and a nucleic acid capable of mediating RNA interference was included in and extended in a manuscript first submitted for publication on March 2, 2004, a few months after the November 24, 2003 filing date of the '176, later published (August 20, 2004) as Chiu, Y.L., Ali, A., Chu, C.Y., Cao, H., & Rana, T.M., Visualizing a Correlation between siRNA Localization, Cellular Uptake, and RNAi in Living Cells, *Chemistry and Biology*, Vol 11, 1165-1175, August 2004 (herein "Chiu, et al., 2004," copy submitted with accompanying Supplementary Information Disclosure Statement) in a section titled "Nanoparticles Effectively Deliver Function siRNA to Cells," page 1169, left column, first full paragraph to page 1171, right column, last full paragraph..

7. The correspondence of the disclosure of the '176 patent application and the Chiu, et al., 2004 publication is summarized in Table 1, below.

Table 1	
'176 Patent Application	Chiu, et al., 2004 Publication
Example 1 (paragraph [0102] of the published application and FIG. 1B)	Page 1169 and Figure 4A
Example 2 (paragraph [0103] of the published application and FIG. 2)	Page 1169 and Figure 4B
Example 7 (paragraph [0111] of the published application and FIGS. 9A –9I)	Page 1170 and Figure 5

8. The results of the same experiments illustrated in FIG. 1B of the '176 application are illustrated in Figure 4A of the Chiu, et al., 2004 publication. The methods used were described as follows (page 1173, right column):

> Cellular Uptake of siRNA by Nanoparticles
>
> HeLa cells were maintained at 37°C in Dulbecco's modified Eagles medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Cells were regularly passaged at subconfluence and plated on 60 mm plates 16 hr before transfection at 70% confluency. 21 nt 5'-Cy3-labeled EGFP sense strand siRNA was deprotected, annealed to unmodified antisense strand siRNA, and purified as described above. As a control, Lipofectamine-mediated transfections were performed as described by the manufacturer for adherent cell lines. For siRNA delivery by nanoparticles, we used a generation four polyamidoamine dendrimer that had 64 surface groups and a diameter of 45 Å. Here, we denote these particles as NP-45. We transfected cells with Cy3-SS/AS-3'N3 EGFP siRNA and NP-45 as described above. Cells were incubated in transfection mixture for 6 hr and washed three times with PBS (Invitrogen) to remove the transfection mixture. Total DNA, RNA, and the transfected Cy3-SS/AS-3'N3 EGFP siRNA were isolated from the cells and subjected to fluorescence measurements on the PTI fluorescence spectrophotometer as described above.

9. The results are described at page 1169, left column:

> We used a generation four polyamidoamine dendrimer that had 64 surface groups and a diameter of 45 Å (reviewed in [49]). Here, we denote these particles as NP-45. We transfected cells with Cy3-SS/AS-3'N3 EGFP siRNA and NP-45 as described above. To evaluate cellular uptake of siRNA, the fluorescence of Cy3-SS/AS-3'N3 EGFP siRNA using NP-45 was normalized to the corresponding Cy3-SS/AS-3'N3 EGFP siRNA fluorescence in cells transfected with 20 µg/ml of Lipofectamine. As shown in Figure 4A, the efficiency of siRNA uptake using 20–40 µg/ml NP-45 (lanes 3 and 4) was almost equal to that measured for 20 µg/ml Lipofectamine (lane 1). However, NP-45 concentrations above this range lowered the siRNA uptake 40%–60% (Figure 4A; lanes 5–8), suggesting that there was a critical concentration range for NP-45-siRNA delivery. Although the total cellular uptake was not significantly changed at higher concentrations of NP-45, we observed a significant change in the cytoplasmic localization and distribution of siRNA (see Figure 5 and section below).

10. The results of the same studies of silencing of CDK9 expression using a delivery medium comprising a dendrimer and a nucleic acid capable of mediating RNA interference that were illustrated in FIG. 2 of the '176 application are illustrated in Figure 4B of the Chiu, et al., 2004 publication, and described as follows:

> Cells transfected with SS/AS-3'N3 CDK9 siRNA using NP-45 (20–200 μg/ml) showed varying degrees of RNAi activity and the highest level of RNAi activity (91%) was observed with 40 μg/ml NP-45 (Figure 4B; lane 3). This maximal RNAi activity correlated with the high siRNA uptake efficiency at the same NP-45 concentration (Figure 4A, lane 4). RNAi efficiencies were decreased (68%–42%) when 100–200 μg/ml of NP-45 were used (Figure 4B, lanes 4 and 5). Higher concentration of NP-45 lowered the cellular uptake (40%–60%) but did not completely inhibit siRNA uptake (Figure 4A, lanes 5–8), suggesting that fraction of active pools of siRNA remained in cytoplasm at 100–200 μg/ml concentrations of NP-45 causing knockdown of CDK9 expression (Figure 4B, lanes 4 and 5). The RNAi activity measured using 400 or 1000 μg/ml NP-45, however, was comparable to mock-treated (no siRNA) cells (Figure 4B; compare lanes 6 and 7 with lane 1). These results were consistent with NP-45 being effective for both siRNA uptake and RNAi activity only within a critical concentration range and further suggested that there was a correlation between siRNA uptake and efficient RNAi activity.

11. The results of transfection of human (HeLa) cells *in vitro* were illustrated in Figure 5 and described as follows:

> The effect of NP-45 on the localization of Cy3-SS/AS-3'N3 EGFP siRNA was assessed after transfecting the Cy3-labeled siRNA and NP-45 (40 μg/ml or 200 μg/ml). siRNA transfected with 40 μg/ml NP-45 also localized to perinuclear regions of the cytoplasm although it appeared to aggregate to more discrete areas within the perinuclear region (Figure 5C). Interestingly, using higher concentration of NP-45 (200 μg/ml), a shift in siRNA localization patterns was observed with cytoplasmic localization appearing more diffuse (Figure 5D). In addition, siRNA was observed in both the nucleus and nucleolus (Figure 5D), indicating that delivering siRNA to cells using NP-45 at this higher concentration altered siRNA subcellular localization. It was not clear how NP-45 at higher concentrations was altering normal siRNA subcellular localization. However, these observations correlated well to the lower RNAi activity associated with using higher concentrations of NP-45 and suggested that subcellular localization of siRNA was important for RNAi.

12. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that

these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 101 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Respectfully Submitted,

Date: 11/6/06

Tariq M. Rana